REMARKS

Claims 3, 5-6, 8, and 10 have been cancelled. Claims 1-2, 11, and 17 have been amended. Claims 1-2, 4, 7, 9, and 11-25 are now pending in this application. Support for the amendments is found in the existing claims and the specification as discussed below. Accordingly, the amendments do not constitute the addition of new matter. Applicant respectfully requests the entry of the amendments and reconsideration of the application in view of the amendments and the following remarks.

Rejection under 35 U.S.C. § 102(b)

Claims 1, 2, 4, 7, and 25 are rejected under 35 U.S.C. § 102(b) as anticipated by Roussaux.

In the last Office Action, the Examiner stated that the arguments were not persuasive because "there is no limitation in the claim, which involves the step of micropropagation" (Paper No. 14, page 2). In response, the claim 1 has been amended to include a micropropagation step as suggested by the Examiner. Support for the amendment of claim 1 is found on page 4, line 28 to page 5, line 7. Support is also found in claim 2 as presented with the amendment of March 10, 2003. Amended claim 1 is now limited to a method for micropropagation.

Applicants assert that the claims as amended are not anticipated by Roussaux. Steps (a) - (f) of claim 1 as amended are not described in a single embodiment, nor under a single heading in Roussaux. In fact one could only come to the method of claim 1 as presented by taking different paragraphs/sentences together from different places out of the 30 pages of the Roussaux document. In order to have anticipation, the elements must be arranged as required by the claim. See *In re Bond*, 910 F.2d 831, 15 USPQ2d 1566 (Fed. Cir. 1990).

Claim 2 now incorporates the limitations of cancelled claim 3. Consequently, Applicants assert that this ground of rejection no longer applies to claim 2. Furthermore, the Examiner has applied no grounds of rejection to claim 3. Consequently, claim 2, which now contains the limitations of claim 3, is believed to be in condition for allowance.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of this ground of rejection is respectfully requested.

Rejections under 35 U.S.C. § 103(a)

Claims 1, 2, and 9 are rejected under 35 U.S.C. § 103(a) as unpatentable over Roussaux in view of Mantell.

The Examiner asserts that Roussaux teaches a method of shoot multiplication using bacteria but does not teach a method wherein shoots are subjected to acclimatization before conventional growing conditions. The Examiner cites Mantell for its teaching on an acclimatization step and asserts that it would have been obvious to modify Roussaux with acclimatization as taught by Mantell.

In response, as stated above, Applicants maintain that Roussaux does not teach a method for micropropagation of plants as presently claimed. Steps (a) - (f) of claim 1 as amended are not described in a single embodiment, nor under a single heading in Roussaux. The teachings of the cited references, taken as a whole, do not teach or suggest the presently claimed invention.

In order to provide a prima facie case of obviousness, the Patent and Trademark Office has the burden to provide a motivation, teaching or suggestion to create the claimed invention. *See, e.g., In re Fine*, 5 U.S.P.Q.2d 1597 (Fed. Cir. 1988). Such motivation, teaching or suggestion is absent in the references cited by the Examiner.

Roussaux never teaches or suggests the use of galls (nodules) as a starting material for propagation. Roussaux merely puts plants with deformed shoots in a solution with antibiotics to see when the pathogen exerts an effect in order to find a solution to the question whether this effect can be compared with the effect *Agrobacterium tumefaciens* has on plants, i.e. whether the galls are the result of a permanent change in the plant itself, which is not the case. Roussaux is very prudent to take this conclusion (e.g. on page 51 " il parait donc possible de conclure....). Propagation is neither taught nor suggested by Roussaux and it is never even suggested by Roussaux that the observed phenomenon would be interesting for producing a larger number of shoots. The Examiner's attention is directed to page 50, under c) conclusion: "les bourgeons des plantes fasciées sont susceptibles de croitre á nouveau lorsquíls sont libérés des constraintes que leur imposent la présence du parasite", i.e. which is similar to the major shoot of the plant, that also encounters effects of bacterial infection but restarts growing after treatment with antibiotics. Also on the same page under b) cultures de nodules bourgeonnonts: "Les nodules porteurs de bourgeons, débarrassés de la bactérie et maintenus en cultures in vitro n'ont pas présenté une prolifiration tissulaire importante. Certain bourgeons se sont par contre notablement allongés et ont donné naissance á des tiges greles et chlorotiques. Les premiers entre-noeuds apparus étaient courts et portaient de petit feuilles mal-formées, mais ultérieurement les tiges ont présenté une morphologie normale". That is, even if one of ordinary skill in the art were motivated to adapt

the teaching of Roussaux to the micropropagation of plants (and Applicants maintain that there is no motivation in the cited references for plant propagation), Roussaux does not provide a reasonable expectation of success that the phenomenon described by Roussaux could be used for plant propagation as the shoots produced by the galls of Roussaux produced thin spindly stems, short malformed leaves, and chlorotic tissue. Thus, there was neither motivation nor expectation of success to apply the teaching of Roussaux to plant propagation.

Furthermore, the present invention relates to a general method that can be used both for monocotyledonous and dicotyledonous plants, as well as for recalcitrant plants that cannot be propagated by in vitro methods. Nowhere in Roussaux, is it described that the pathogen has such a broad host range. The present inventors have found that fasciating microorganisms such as *Rhodococcus* can be used as a universal tool to propagate any plant that reacts on the pathogen. The present specification exemplifies plant propagation using *Rhodoccocus fascians* for diverse plant species including *Nicotiana tabacum*, *Papaver somniferum*, *Artemisia annua*, *Vigna unguiculata*, *Atropa belladona*, *Sesbania rostrata* and *Digitalis lanata* (see present specification, page 13, lines 9-11). This host range for gall producing bacteria could not have been predicted from Roussaux and was totally unexpected.

Further, Roussaux clearly states that the abnormal shoot formation only results from existing meristems, e.g. page 41 "le C. fascians nágit que sur des tissus á caractére méristimatique,..." and further on page 51, in the context of "nodules feuillés" (leafy galls): "C. fascians n'exerce aucune action néoformatrice de meristémes caulinaires". Thus, according to Roussaux, if there are no meristems, no effect of the bacteria is to be expected. Applicants have found that this is not true. In addition to existing meristems, *C. fascians* will also induce de novo meristem formation, starting from corticoid cells, on the veins of the leaves or in the leaf's axil. Applicants' have shown induction of leafy galls on the leaves (at the border or on the veins). This has also been published by the present inventors (Carmem-Lara de O. Manes, et al., Mol Plant Microbe Interact, vol 14, no. 2, pages 189-195, February 2001). See Attachment A. This not only means that shoots can be amplified from existing shoots, but also that the shoots can be de novo made from differentiated cells. The presently claimed invention relates to any plant material and includes leaf discs, organs, tissue fragments, isolated cells, protoplasts and cell cultures as well as meristematic tissue such as seedlings, seeds and embryos (claim 1). This result was again totally unexpected in view of the cited references.

Mantell, et al. are cited for teaching acclimatization. Mantell et al is a textbook teaching on clonal propagation of plants. However, Mantell et al. do not teach the use of a microorganism that induces fasciation. Thus, Mantell, et al. fail to correct the deficiencies of Roussaux, discussed above.

Claim 2 now incorporates the limitations of cancelled claim 3. Consequently, Applicants assert that this ground of rejection no longer applies to claim 2. Furthermore, the Examiner has applied no grounds of rejection to claim 3. Consequently, claim 2, which now contains the limitations of claim 3, is believed to be in condition for allowance.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of this ground of rejection is respectfully requested.

Claims 17-24 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Roussaux in view of Bhojwani et al. and Mantell.

The Examiner asserts that Roussaux teaches a method of shoot multiplication using bacteria but does not teach a method wherein shoots are subjected to growth limiting conditions after multiplication. However, the Examiner cites Bhojwani, et al. for their teaching on plant germplasm storage and Mantell for its teaching on acclimatization of plants and asserts that it would have been obvious to modify Roussaux with the teachings of Bhojwani, et al. and Mantell.

Neither Roussaux nor Mantell, et al. teach or suggest the claimed invention as discussed above. Bhojwani, et al. do not correct the deficiencies of Roussaux and Mantell, et al.

Motivation to use a fasciating microorganism such as *R. fascians* in such a method of micropropagation is also not found in Bhojwani, et al. which is directed to cryopreservation. Applicants assert that one of ordinary skill in the art would not be motivated to turn to a teaching on cryopreservation to modify the teaching of Roussaux. While use of low temperature in tissue preservation was known, there is no motivation provided by the Examiner from the cited references to include a low temperature step in a method of micropropagation. Furthermore, there is no teaching provided in any of the cited references on the use of leafy galls or shoot outgrowths produced by fasciating microorganisms as a vehicle for storage of plant germplasm.

Furthermore, claims 17-24 are now limited to fasciation-inducing factors that comprise the *fas* products and/or the *att* products encoded by the pFiD188 plasmid. Neither the *fas* products nor the *att* products are taught by any of the cited references. Thus, the combination of references does not teach or suggest all claim limitations.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of this ground of rejection is respectfully requested.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 11-16 are rejected under 35 U.S.C. § 112, first paragraph as containing subject matter which is not described in the specification in such a way so as to enable one skilled in the art to which it pertains to make and/or use the invention.

The Examiner asserts that claims 11-16 are not enabled because of the recitation of factors which are very broad in scope and gene products/proteins which are unknown.

This ground of rejection is believed to be overcome by Applicants' amendment of claim 11 to *fas* products and *att* products encoded by the pFiD188 plasmid. As the claims are now limited to these loci which are described in the specification, particularly at page 7, line 4 to page 11, line 37, it would not amount to undue experimentation to practice the invention as presently claimed and limited to *fas* and *att* products.

Regarding the nature of the invention, the invention of claim 11 is drawn to contacting plant material with one or more fasciation-inducing factors. The scope of the invention has been narrowed significantly to the *fas* and/or *att* products. The specification provides sufficient guidance through its description, particularly at page 7, line 4 to page 11, line 37. Working examples are provided indirectly through the use of *Rhodococcus fascians* strain D188 in the examples at pages 13-15. Protein production from the loci is described in the specification. See for example, page 8, lines 16-31. Thus, given that the claims are now limited to *fas* and *att* and that these loci are described at length in the specification, the amount of experimentation required by one skilled in the art would not be undue.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

Claims 17-24 are rejected under 35 U.S.C. § 112, first paragraph, because the specification, while being enabled for methods using microbes themselves, does not reasonably provide enablement for methods using factors. The Examiner asserts that the specification is not enabling for methods using fasciation factors.

Applicants have amended claims 17-24 to *fas* products and *att* products encoded by the pFiD188 plasmid. As the claims are now limited to these loci which are described in the specification, particularly at page 7, line 4 to page 11, line 37, for the reasons given above for the rejection of claims 11-16 under 35 U.S.C. § 112, first paragraph, it would not amount to undue experimentation to practice the invention as presently claimed and limited to *fas* and *att* products.

In view of Applicants' amendments and arguments, reconsideration and withdrawal of the above ground of rejection is respectfully requested.

CONCLUSION

In view of Applicants' amendments to the claims and the foregoing Remarks, it is respectfully submitted that the present application is in condition for allowance. Should the Examiner have any remaining concerns which might prevent the prompt allowance of the application, the Examiner is respectfully invited to contact the undersigned at the telephone number appearing below.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Sept. 2, 2003

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ATTACHMENT A

MPMI Vol. 14, No. 2, 2001, pp. 189–195. Publication no. M-2000-1127-02R.

De novo Cortical Cell Division Triggered by the Phytopathogen *Rhodococcus fascians* in Tobacco

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Plant growth, development, and morphology can be affected by several environmental stimuli and by specific interactions with phytopathogens. In many cases, plants respond to pathogenic stimuli by adapting their hormone levels. Here, the interaction between the phytopathogen *Rhodococcus fascians* and one of its host plants, tobacco, was analyzed phenotypically and molecularly. To elucidate the basis of the cell division modulation and shoot primordia initiation caused by *R. fascians*, tobacco plants were infected at leaf axils and shoot apices. Adventitious meristems that gave rise to multiple-shoot primordia (leafy galls) were formed. The use of a transgenic line carrying the mitotic *CycB1* promoter fused to the reporter gene coding for β-glucuronidase from *Escherichia coli* (*uidA*), revealed that stem cortical cells were stimulated to divide in an initial phase of the leafy gall ontogenesis. Local cytokinin and auxin levels throughout the infection process as well as modulation of expression of the cell cycle regulator gene Nicta;*CycD3;2* are discussed.

Additional keywords: cyclinD3; epiphylly; phytohormones.

The phytopathogenic gram-positive bacterium *Rhodococcus fascians* infects a wide range of monocotyledonous and dicotyledonous plants causing several types of malformations. A 200-kb linear plasmid is responsible for pathogenicity and contains virulence genes, one of which codes for an isopentenyl transferase (IPT) homolog that is putatively involved in cytokinin biosynthesis (Crespi et al. 1992).

The effects caused by *R. fascians* vary from a fasciated phenotype to the initiation of multiple-shoot primordia accompanied by the suppression of shoot outgrowth, resulting in a leafy gall. The phenotypes depend on the host and the age of the plant as well as on the inoculation method and site of infection. *Nicotiana tabacum* (L.) and *Arabidopsis thaliana* (L.) Heynh. were used as model plants to investigate morphological aspects of *R. fascians* infection. When tobacco plants are decapitated and infected, multiple lateral shoots are formed at the site of the wounding that give rise to a leafy gall (Vereecke et al. 2000). Morphological changes observed after vacuum infiltration of *A. thaliana* include increased number of flowers, early flowering, outgrowth of axillary meristems, overall stunted phenotype as well as abnormal flower formation, and altered leaf shape (T. M. Ritsema, C.-L. de O. Manes, K. Goethals, and M. Holsters, *unpublished*).

Plant morphogenesis relies essentially on cell division and expansion because plant cells do not migrate. In mature plants, cell division is restricted to meristematic regions. Shoot and root meristems show specific patterns of cell division that are controlled genetically (Meyerowitz 1997). Nevertheless, most plant cells are capable of changing their fate in response to appropriate stimuli. Cell division patterns and rates are affected by different factors such as temperature, nutrient and water availability, and pathogen attack (Ben-Haj-Salah and Tardieu 1995; Dudley et al. 1987; Gastal and Nelson 1994; Sacks et al. 1997).

Recently, many efforts were made to obtain a clear picture of the eukaryotic cell cycle machinery and its regulation. Cell-cycle progression can be arrested at different phases and reactivated through the induction of regulatory proteins. The key components are the cyclins and the cyclin-dependent kinases (CDKs). Cell division is regulated by the activation of CDKs through the binding of cyclins. In plants, several genes that encode cell-cycle regulators have been isolated, and their expression pattern and/or associated protein activity investigated (Mironov et al. 1999). Several studies demonstrated that nutrients, cytokinins, and auxins up regulate D-type cyclins and A-type CDK transcripts (Carle et al. 1998; De Veylder et al. 1999; Fuerst et al. 1996; Tréhin et al. 1998). Additionally, endogenous cytokinin, in particular zeatin, is required for cell-cycle progression in BY_2-cultured cells (Laureys et al. 1998; Redig et al. 1996). Recently, Riou-Khamlichi et al. (1999) observed that the *CycD3* transcript is up regulated by zeatin and its overexpression triggers the formation of cytokinin-independent calluses. In addition to a role in cell division, cytokinins interfere in cell expansion, releasing axillary bud dormancy and delaying leaf senescence. Altered endogenous cytokinin levels and their relation to morphological changes have been investigated extensively by the genetic engineering of different plant species (Li et al. 1992; McKenzie et al.

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1994; Medford et al. 1989; Roeckel et al. 1998; Schmülling et al. 1993). The overall morphological changes caused by *R. fascians* on its hosts and the presence of an *IPT* gene on the linear plasmid suggest that cytokinins might play an important role in symptom development. Although several virulence genes of *R. fascians* have been identified to date (Crespi et al. 1994), the signaling for pathogenicity is still unknown. Here, we study leafy gall ontogenesis, starting at the initiation of cell division in tobacco stem cortical cells triggered by *R. fascians* infection. The endogenous hormone levels throughout the infection process as well as the modulation of cell-cycle gene expression are evaluated and discussed.

RESULTS

Spot inoculation assay.

Different methods were used to infect host plants with *R. fascians* such as decapitation site infection, vacuum infiltration, and watering (Vereecke et al. 2000). The symptoms displayed by the infected plants were not homogeneous and, except

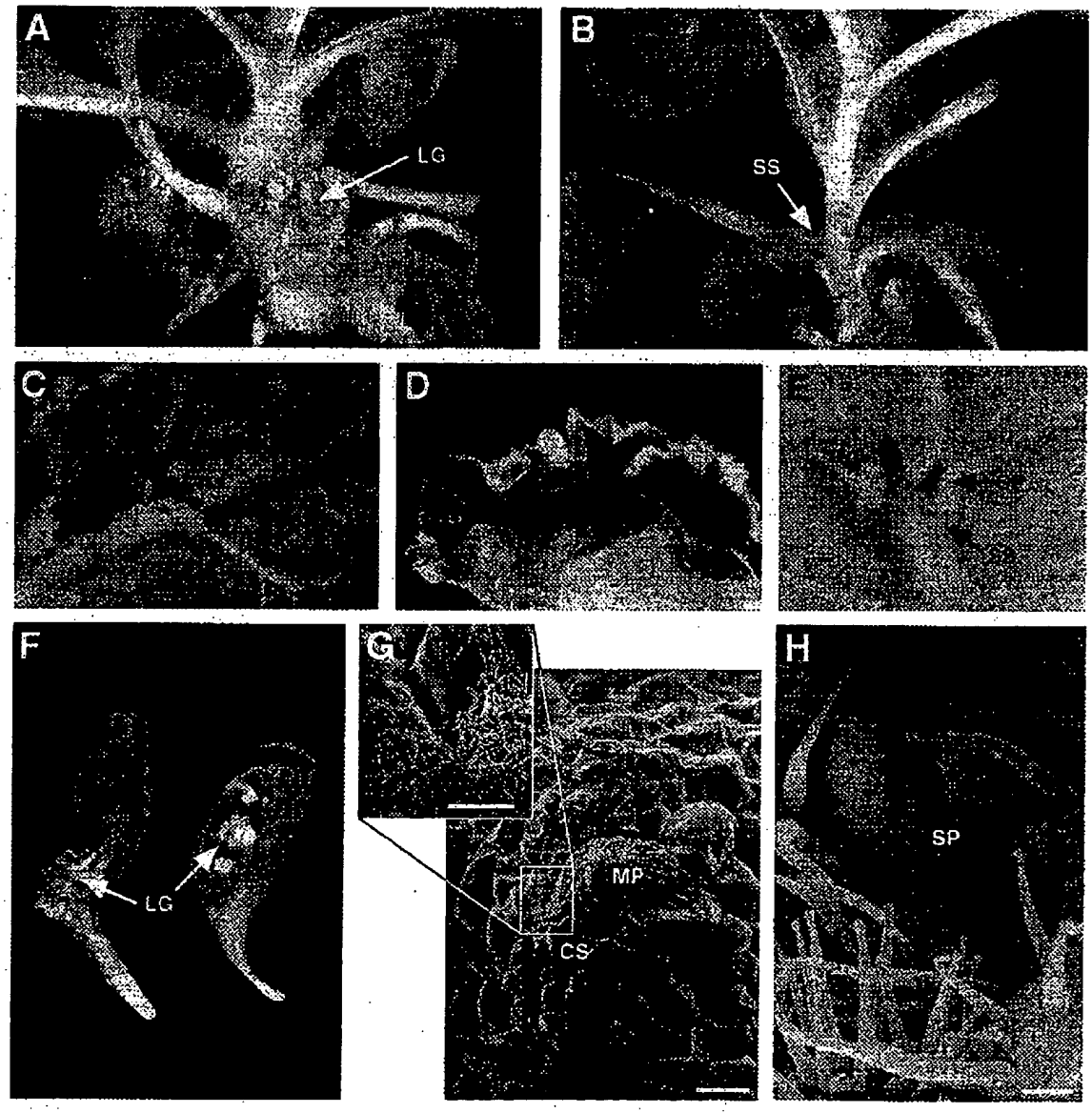

Fig. 1. Tobacco phenotypes in response to *Rhodococcus fascians* spot inoculations. **A,** Leafy gall derived from an infected leaf axil. **B,** Control plant infected with the nonvirulent strain D188-5 of *R. fascians*. **C and D,** Epiphyllous shoots on the leaf blade and leaf margin. **E,** Shoot primordium arising from locally spotted site. **F,** Leafy gall originated from (E). **G,** Scanning electron micrograph of an infected leaf midrib showing epidermal cell swelling and meristem primordium. Detail of colonization site of *R. fascians* surrounding meristem primordium. **H,** Scanning electron micrograph of an adventitious shoot primordium originated from a leaf blade spot infection. CS = colonization site; LG = leafy gall; MP = meristem primordium; SP = shoot primordium; SS = spot site. Bars = 100 µm (**G and H**) and 10 µm (inset).

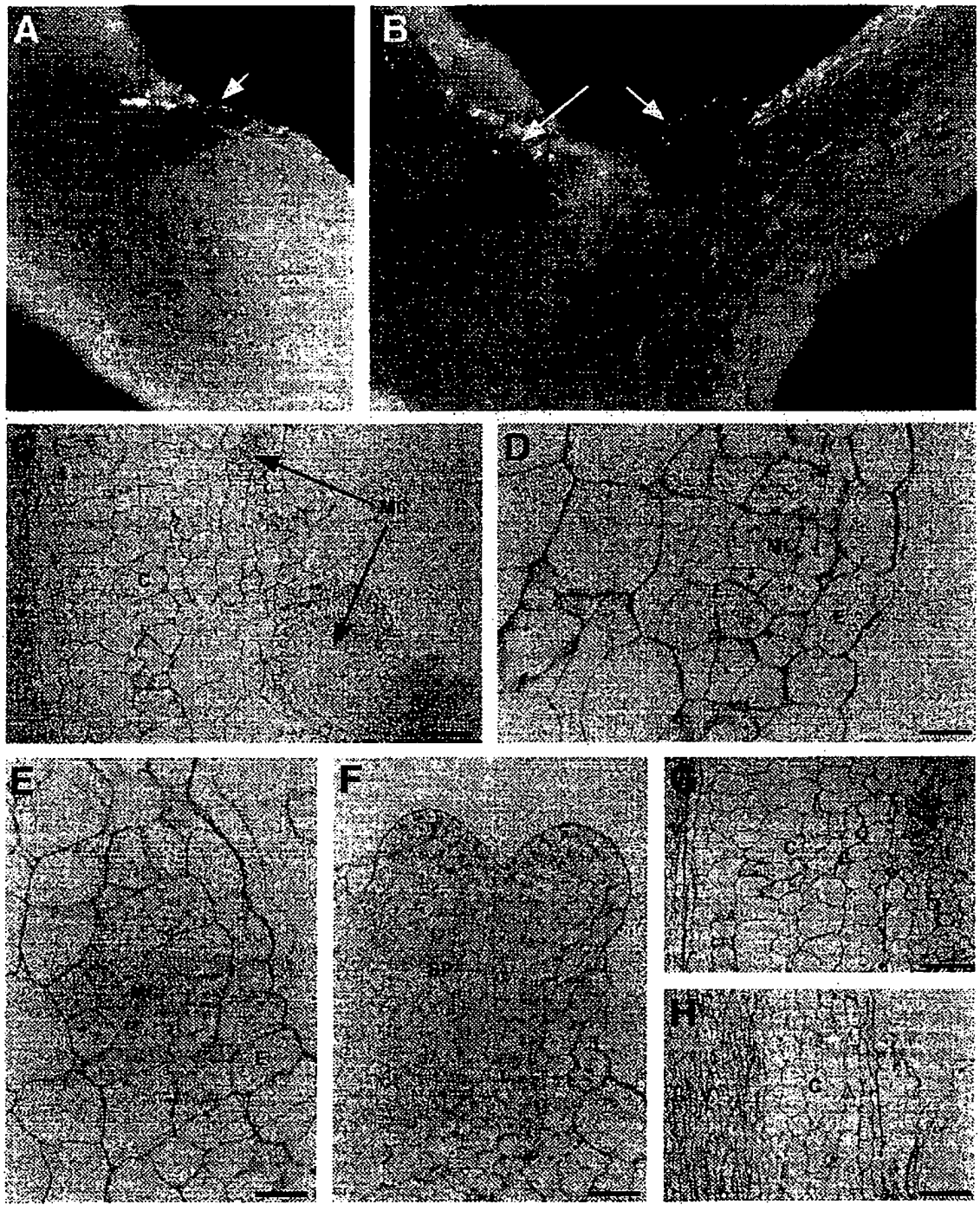

Fig. 2. *CycB1;1* promoter activity and microscopic analysis of a tobacco transgenic line spot-inoculated with *Rhodococcus fascians*. **A and B,** *GUS* expression indicates dividing cells (arrows) after inoculation of leaf axils. **C,** Longitudinal sections of an inoculated axil show mitotic centers and a shoot primordium originating from the outer cortical cells of the stem. **D,** Details from cortical cell division initiation. **E and F,** Shoot primordium formation. **G,** Overview of cortical cells from a spot-inoculated plant. **H,** Noninfected cortical cells. C = cortex; E = epidermis; MC = mitotic center; SP = shoot primordium; V = vascular tissue. Bars = 200 μm (**C, G, and H**) and 50 μm (**D to F**).

for decapitated plants, these methods were not adequate to obtain localized responses. To avoid the above-mentioned drawbacks, a localized, nondamaging infection method was developed. Leaf axils of tobacco plants were inoculated with a droplet of a log-phase culture of *R. fascians*. After 1 week, these areas invariably formed meristem initials that developed into a leafy gall 4 weeks later (Fig. 1A and B). No positional preference from which axil (younger-older) responded to spot inoculation was observed. Spot inoculation also was performed on the leaf blade and the shoot apex. Invariably, when the shoot apex was infected, developing leaves displayed epiphyllous shoots along the vasculature and sometimes at the margins (Fig. 1C and D). For the leaf blade spot inoculation, the frequency of responses varied according to the developmental stage and spotting site. Young leaves responded better, and infection at the leaf pedicel and midrib produced more reproducible responses, leading to activation of adventitious meristems (Fig. 1E) and leafy gall formation (Fig. 1F).

R. fascians colonizes the surface and internal spaces of the leaf tissue, but colonization is not always related to symptom development because virulent and nonvirulent strains have comparable surface-colonization capacities (K. Cornelis, T., Ritsema, J. Nijsse, M. Holsters, K. Goethals, and M. El Jaziri, *unpublished*). Scanning electron microscopy was performed on the infected tissues to investigate whether newly formed structures contained bacteria. Invariably, in the vicinity of leaf bladeborne shoots, scattered bacterial clusters were found but occurred rarely on the newly formed shoots themselves (Fig. 1G and H). The same observation was made when petiole and stem-derived adventitious shoots were analyzed. Therefore, we can suggest that shoot primordia formation is restricted to colonization sites.

From cortical cell division to leafy gall ontogenesis.

Morphological analysis of tobacco plants infected by *R. fascians* suggests that cell division and expansion are affected. To observe the first events induced by *R. fascians*, a transgenic line of tobacco carrying the mitotic *CycB1;1* promoter-*gus* fusion was used (Ferreira et al. 1994). The *CycB1;1* promoter is active at the G2-to-M transition and during mitosis (Shaul et al. 1996). Segments of stems comprising infected areas were harvested 1, 3, 5, 7, and 14 days after spot inoculation at leaf axils. The first visible symptom was a thickening of the stem 4 days after infection. Actively dividing cells, as shown by GUS staining, appeared 4 to 7 days after infection (Fig. 2A and B). Serial, longitudinal thin sections were stained and scored for the presence of mitotic figures and early cell-division events. At day 1 and 3 after infection, no major morphological changes were found in infected tissues compared with noninfected tissues or tissues infected with an nonvirulent strain of *R. fascians*. Seven days postinoculation, mitotic figures were observed in the outer cortical cells of stems infected with wild-type bacteria (Fig. 2C to E). In this case, cell-division events always correlated with a faint GUS staining in the ultrathin sections. The dividing cells were reoriented to form a shoot meristem that developed a leaf primordium and later into a shoot with inhibited outgrowth (Fig. 2F). At the infection site, inner and outer cortical cells of the stem were enlarged (Fig. 2G and H).

Cytokinin and auxin contents of infected areas.

Cell division and differentiation are controlled by hormones. To correlate activation of cortical cell division with altered hormone levels at the site of infection, plants were spot inoculated with virulent and nonvirulent strains (control plants) of *R. fascians* at the leaf axils. One, 2, and 3 weeks postinoculation, stem segments comprising the infected axils were harvested for local determination of cytokinins and indole-3-acetic acid (IAA) levels by immunoaffinity chromatography with a broad-spectrum antibody (Prinsen et al. 1995a; Prinsen et al. 1998). The data obtained indicated that the measurement levels of cytokinin bases (zeatin, dihydrozeatin, and isopententenyl adenine) or their derivatives (ribotides, glucosides, and phosphates) were too low to be significant (data not shown). In contrast, during the first 2 weeks of the infection process, IAA levels were higher in tissues inoculated with the virulent strain of *R. fascians* than in control tissues: 89.4 and 224.0 pmol of IAA per g (fresh weight) was obtained compared with 56.4 and 146.0 pmol of IAA per g in control tissues 1 and 2 weeks postinoculation, respectively.

***CycD3;2* transcript levels are enhanced by *R. fascians* infection and by cytokinins.**

D-type cyclin genes were isolated from tobacco and their expression patterns during cell cycle were investigated by Sorrell et al. (1999), who showed that the Nicta;*CycD3;2* transcripts were induced rapidly as stationary cells reentered the cell cycle. To evaluate the effect of *R. fascians* inoculation on Nicta;*CycD3;2* expression, a RNA gel blot analysis was performed with RNA from spotted axils with Nicta;*CycD3;2* as a hybridization probe. The results showed that 5 days postinfection, the *CycD3;2* transcript levels increased up to fourfold when compared with control material infected with the nonpathogenic strain (Fig. 3A). The tobacco *CycD3;2* also was induced in seedlings treated with 1 μM zeatin for 24 h (Fig. 3B).

DISCUSSION

We used a nondamaging infection assay to investigate the *R. fascians*-host interaction at responsive tissues such as leaf axils, leaf blade, and shoot apex. The homogenous responses obtained were the activation of axillary and adventitious meristems that developed in a structure defined as leafy gall. The ontogenesis of this particular symptom has not been described previously. We show that the initial step of leafy gall formation is de novo cell division in the outer cortical cells of stems. Dividing cells form a meristem that acquires zonation and develops into a shoot primordium. Interestingly, a parallel can be made with the induction of nitrogen-fixing root nodules in the *Rhizobium*-legume symbiosis. There, upon stimulation by nod factors, root cortical cells dedifferentiate and start to divide, establishing a nodule primordium (Yang et al. 1994). Signals derived from *R. fascians* induce a mitosis in the outer cortical cells of the stem. Because no evidence for enhanced levels of common cytokinins or cytokinin derivatives during the infection process could be obtained, the hypothesis proposed is that novel types of bacterially produced cytokinin analogs are involved in this process. A previously reported enhancement in IAA levels of infected plants (Vereecke et al. 2000) could be confirmed by a more sensitive technique. However, the origin of the auxin is not clear: it can be correlated with the formation of multiple meristems, which are known sources of auxin production in plants, or with bacterially produced auxins.

The shoot outgrowth in the leafy gall is inhibited, possibly as a result of an extreme apical dominance exerted by each lateral shoot primordium over its neighbors. When a leafy gall is set free from bacteria by antibiotic treatment and is placed on growth medium, normal shoots develop to form a phenotypically normal plant (Vereecke et al. 2000). These data corroborate Lacey's (1936) statement that the presence of the bacteria is crucial for symptom maintenance. Here, symptom development is correlated with the presence of bacteria by the microspotting assay, and responsive colonization sites develop into a leafy gall structure, whereas the rest of the plant continues to grow normally.

Another feature of the system is the induction of epiphyllous shoots. Few data are available on adventitious meristem activation in planta. Naturally occurring epiphyllous shoots are found in the ornamental plant *Kalanchoe daigremontiana* and are thought to arise from leaf margin cells that are blocked in the G1 phase. These cells are reactivated to form undifferentiated meristems that further develop into a small shoot (Kerstetter and Hake 1997). Epiphylly has been observed in plants that overexpress members of the *Kn1* class of plant homeobox genes and in plants overexpressing a cytokinin synthesis gene (*ipt*) from *Agrobacterium tumefaciens* (Chuck et al. 1996; Li et al. 1992; Sinha et al. 1993). Recently, Rupp et al. (1999) showed a direct link between cytokinin overproduction in transgenic *ip* overexpressing *Arabidopsis* spp. lines and increased mRNA steady-state levels of *KNAT1* and *STM* homeobox genes. Both gene transcripts also are enhanced in the cytokinin overproducing shoot meristem mutant *amp1*. Together these findings suggest that homeobox gene overexpression and cytokinin overproduction act in the same developmental pathway.

The main advantage of the *R. fascians*-tobacco system relies in the fact that locally infected plant tissues respond to the bacterial stimuli, thus providing homogenous and specific material to investigate aspects of shoot development at the physiological and molecular level.

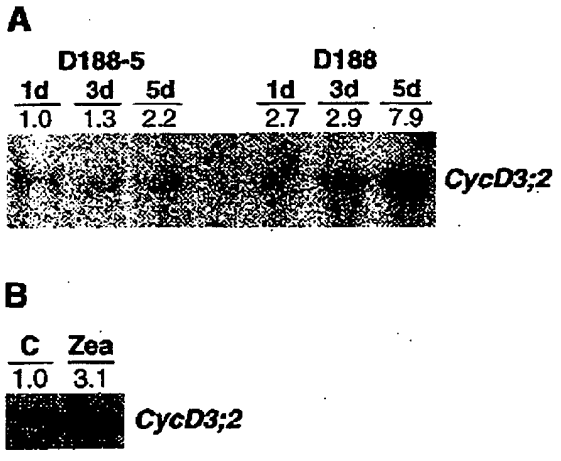

Fig. 3. Nicta;*CycD3;2* RNA induction by *Rhodococcus fascians* and cytokinin. **A**, Tobacco spot-infected axils 1, 3, and 5 days postinoculation with *R. fascians* virulent (D188) and nonvirulent (D188-5) strains. **B**, Tobacco seedlings grown 7 days in liquid Murashige and Skoog (MS) medium and treated 24 h with MS alone (C) or supplemented with 1 μM zeatin (Zea). Gray density values (1.0-7.9) indicate approximate inducibility.

Currently, cell-cycle markers are used to study the molecular basis of plant responses toward environmental factors such as stress, pathogens, nutrients, light, and hormones. In the field of plant-microbe interactions, these genes have been used to study nodule formation upon *Rhizobium* sp. infection (Goormachtig et al. 1997; Yang et al. 1994) and the formation of giant cells and syncytia caused by nematodes (De Almeida Engler et al. 1999; Niebel et al. 1996). Cell-cycle markers proved to be a useful tool to analyze the initial stages of the *R. fascians*-tobacco interaction. By assaying the *CycB1;1* promoter activity, we observed the first cell-division events 3 days after infection. The main result was the correlation of the *CycD3;2* transcript inducibility by *R. fascians* stimuli. D-type cyclins are involved in the regulation of the G1-to-S transition. When stimulated to reenter the cell cycle, quiescent cells show enhanced transcript levels of D-type cyclin genes (Soni et al. 1995). For what we believe is the first time in planta, we show the enhancement of *CycD3;2* transcript levels triggered by a phytopathogen. Our data indicate that signals of *R. fascians* are capable of reprogramming cells to reenter the cell cycle in a controlled, patterned way because the final result is the formation of a well-defined structure, the leafy gall.

MATERIALS AND METHODS

Plant material and growth conditions.

N. tabacum (L.) cv. (W38) and Petit Havana (SR1), including the transgenic p*CycB1;1-gus* line (Ferreira et al. 1994), were grown in vitro on half-strength Murashige and Skoog (MS) (1962) medium under a 16-h photoperiod at 24 ± 2°C.

Bacterial strains.

R. fascians strains D188 (pathogenic) and D188-5 (a plasmid-free, nonpathogenic strain) (Desomer et al. 1988) were grown in solid or liquid YEB medium (Miller 1972) at 28°C.

Spot infections.

Bacteria from a late, exponential 2-day-old culture were centrifuged, washed, and resuspended in 0.2 M phosphate buffer (pH 5.7) containing 0.05% Na-citrate, 0.025% $MgSO_4$, and 0.001% thiamine. Aliquots (0.5 to 2 μl) of the bacterial suspension were applied with a glass micropipette that was eventually sustained by a micromanipulator on the target areas.

Histochemical GUS assays.

The histochemical GUS assays were carried out according to Jefferson (1987), with some modifications described by Hemerly et al. (1993). Sections were observed and photographed under a Stemi SV11 stereomicroscope (Zeiss, Jena, Germany).

Microscopic analysis.

Plastic embedding by Technovit 7100 resin (Kulzer Histo-Technik, Wehrheim, Germany) and sectioning were performed according to the manufacturer's protocol. Serial sections (5 μm) were stained with ruthenium red (Sigma, St. Louis, MO, U.S.A.) 0.05% wt/vol water solution and mounted with Depex mounting medium (Gurr, Poole, England). Sections were photographed under a Diaplan light microscope (Leitz, Heerbrugg, Switzerland). Scanning electron microscopy was performed according to Vereecke et al. (2000).

Nicta;*CycD3;2* probe synthesis.

First-strand cDNA from total RNA extracted from *N. tabacum* cv. (W38) stems was synthesized with the SuperScript preamplification system kit (GIBCO-BRL, Gaithersburg, MD, U.S.A.). One-tenth of the reaction mixture was used for the PCR amplification of a fragment from the tobacco *CycD3;2* gene (Sorrell et al. 1999) in 50 µl of PCR mixture containing 1.5 mM $MgCl_2$, 1× PCR buffer II (Perkin Elmer, Norwalk, CT, U.S.A.), all four deoxynucleotide triphosphates (0.2 mM each), 0.2 µM of each primer (F and R), and five units of AmpliTaq (Perkin Elmer). The oligonucleotides used as primers were designed according to sequence information (GenBank accession no. AJ011894) by the SeqLab-prime computer program, version 10.0 (Genetics Computer Group, Madison, WI, U.S.A.): F, forward (5′-GGAAAATGAATCCAGTGACACC-3′) and R, reverse (5′-CCGACCACGAATCATTACAG-3′). Thirty-five cycles at 95°C for 30 s, 55°C for 45 s, 72°C for 3 min, and a final extension of 5 min were performed. A fragment of the expected 420-bp size and sequence was purified from an agarose gel with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and used to prepare radiolabeled DNA probes with the T7 QuickPrime system (Amersham Pharmacia Biotech, Little Chalfont, U.K.).

RNA analyses.

Total RNA was isolated with the RNeasy plant mini kit (Qiagen), according to the manufacturer's protocol. Twenty-five micrograms of RNA samples per lane were separated on 1.2% agarose-formaldehyde gel and transferred to Hybond-N filters (Amersham Pharmacia Biotech). RNA blots were stained with methylene blue to confirm equal loading. Filters were hybridized for 16 h with radiolabeled DNA probes at 65°C in hybridization buffer (100 mM phosphate buffer, pH 7.2; 7% sodium dodecyl sulfate; 0.5 mM EDTA) with 1% bovine serum albumin and 20 µg of denatured herring sperm per ml and washed for 15 min with 0.5× hybridization buffer once at room temperature and twice at 65°C. Hybridized filters were exposed in a PhosphorImage cassette (Amersham Pharmacia Biotech) for 10 days.

Hormone analyses.

Cytokinins and IAA were purified and measured as described previously (Prinsen et al. 1995a; Prinsen et al. 1995b). Cytokinins were extracted overnight from 0.5 g of frozen tissue in $CHCl_3$–methanol (MeOH)–water–acetic acid (Bieleski 1964) and purified by combining solid-phase extraction and immunoaffinity chromatography with a broad-spectrum anti-cytokinin antibody. The 20 ng each of stable isotopes $^2H_5$-Z, $^2H_5$-[9R]Z, $^2H_5$-[9G]Z, $^2H_5$-[7G]Z, $^2H_5$-(OG)Z, $^2H_5$-(OG)[9R]Z, $^2H_5$-[9R]Z-phosphate, $^2H_6$-iP, $^2H_6$-[9R]iP and, $^2H_6$-[9G]iP (Apex International, Honiton, U.K.) were added as an internal tracer for recovery and analytical purposes. The different cytokinin fractions obtained after purification were analyzed by (+)ES micro LC/LC-MRM-MS/MS (Prinsen et al. 1995a; Prinsen et al. 1998). IAA was extracted overnight from 0.5 g of frozen tissue in 80% MeOH (9 ml of fresh weight per g) and 50 ng of (phenyl-$^{13}C_6$-)-IAA (Cambridge Isotope Laboratories, Andover, MA, U.S.A.) was initially added as internal tracer for recovery and analytical purposes. After pentafluorobenzyl derivatization of IAA, pentaflurobenzyl-IAA was analyzed by negative-ion chemical ionization GC-SIR-MS (Epstein and Cohen 1981). Prior to purification, IAA conjugates were converted to free IAA by alkaline hydrolysis (Bialek and Cohen 1989). The data presented correspond to the average of 20 to 30 pooled infected axils in one experiment.

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